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New small-scale cross-axis coil planet centrifuge The design of the apparatus and its application to counter-current chromatographic separation of proteins with aqueous–aqueous polymer phase systems

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Abstract

The cross-axis coil planet centrifuge (*X*-axis CPC) is useful for partitioning macromolecules with aqueous–aqueous polymer phase systems. The floor model we have built with a pair of separation columns had some shortcomings such as requirement of large space, short life of the flow tubes, and difficulty in installing columns. In order to improve the partition efficiency and the utility of counter-current chromatography (CCC), a new small-scale *X*-axis CPC was designed and fabricated in our laboratory. The down-sizing of the apparatus was done by reducing the scale to about 1/2 of our original model of X-1.5 L type with several improvements. Performance of the apparatus was evaluated on protein separation using an aqueous–aqueous polymer phase system composed of polyethylene glycol 1000 and dibasic potassium phosphate with four multilayer coiled columns. A series of experiments revealed that the combination of right- and left-handed coils produced the best partition efficiencies for both lower and upper mobile phases by selecting the revolution direction. The overall results indicate that the head–tail elution mode substantially affects to the peak resolution and stationary phase retention. This new *X*-axis CPC would be useful for the separation of various kinds of biologically active compounds.

Keywords: Counter-current chromatography; Instrumentation; Cross-axis coil planet centrifuge; Proteins; Polymer phase system

1. Introduction

Counter-current chromatography (CCC) is a type of liquid–liquid partition chromatography with the unique feature of eliminating the use of a solid support matrix. Among many CCC systems developed in the past, the type-J multilayer coil planet centrifuge (type-J multilayer CPC) and the cross-axis coil planet centrifuge (X-axis CPC) have proven most efficient among all other CCC models [1–4]. 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 twice about its own axis (which is parallel to the central axis) in the same direction when viewed by an observer outside the system. On the other hand, the *X*-axis CPC produces a synchronous planetary motion of the column in such a way that it revolves around the vertical central axis of the centrifuge, while rotating about its horizontal axis at the same angular velocity [5,6]. The difference in the planetary motion between these two instruments provides distinctive behavior of the two-phase solvent systems.

The type-J multilayer CPC performs excellent separation with organic–aqueous two-phase solvent systems, whereas the *X*-axis CPC is used for highly polar two-phase solvent systems, such as aqueous–aqueous polymer phase systems.

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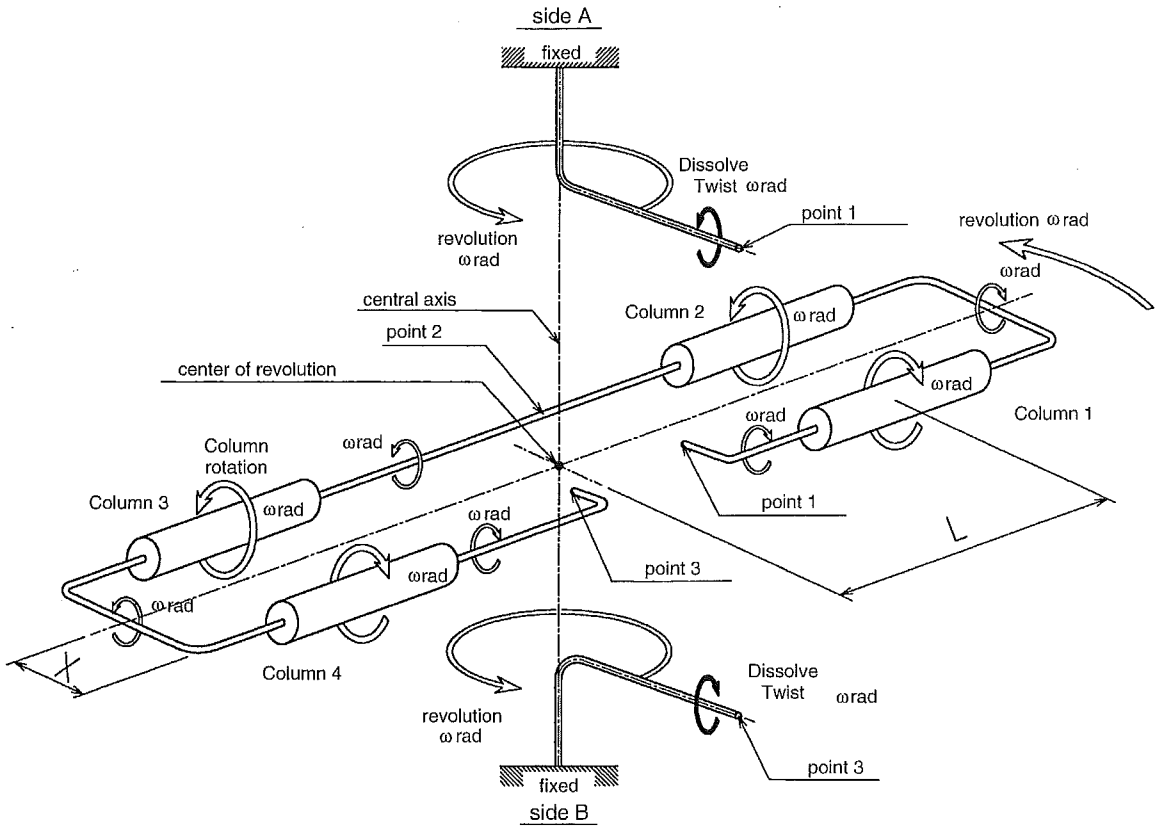

Fig. 1. Principle of the new design of a twist-free single-line flow tube layout. Counter-clockwise revolution is shown.

Among a series of previous experiments, it was revealed that the ratio between the revolution radius (X), which corresponds to "R"-value in the type-J multilayer CPC, and the lateral deviation (L) (see Fig. 1) of the column is an important parameter in retention of the stationary phase in various types of the X-axis CPC including X [5,6], XL [7], XLL [8], and XLLL [9]. Those studies have shown that an increase in ratio L/X results in high retention of the stationary phase by reducing the phase mixing effect. Consequently, X-axis CPCs can provide sufficient retention of the stationary phase of viscous, low-interfacial tension polymer phase systems useful for the separation of proteins.

In this connection, we have previously developed a versatile design of the X-axis CPC, which can accommodate a pair of column holders in two different positions ($L/X = 1.5$ and $X = 0$), which allow the separation of proteins using aqueous–aqueous polymer phase systems [10–12]. Our previous studies revealed that high partition efficiency was attained with a pair of multilayer coils or eccentric coil assemblies in the off-center position. This apparatus is also useful for the separation of highly polar compounds such as sugars [13], hippuric acid and related compounds [14], vitamins [15,16], and water-soluble organic acids [17]. However, down-sizing of the apparatus was required to improve the partition efficiency as well as its utility.

The present paper describes the design and fabrication of our new small-scale X-axis CPC of an X-1.5L type and its application to evaluate the partition efficiency on protein separation with aqueous–aqueous polymer phase systems.

2. Experimental

2.1. Apparatus

The small-scale X-axis CPC employed in the present studies was newly constructed at the Machining Technology Center of Nihon University, Chiba, Japan. The design and fabrication of the apparatus are described in detail in Section 3 below.

2.2. Preparation of coiled column

Each multilayer coil was prepared by tightly winding a piece of 1.0 mm I.D. and 2.0 mm O.D. PTFE (polytetrafluoroethylene) tubing (Flon Kogyo, Tokyo, Japan) around the holder hub of 3 cm in diameter, forming five tight-coiled layers between a pair of flanges spaced 5 cm apart. Each coiled column was prepared according to the following procedure: The tubing was directly wound onto the holder hub starting on the proximal (gear) side (close to the center of revolution). After each coil layer was completed, the layer was wrapped with an adhesive tape and the tubing was straightly returned to the other side to resume winding in the same direction. It results in a multilayer coil assembly composed of either entirely right- or left-handed coils, which is different from that commonly used in the type-J multilayer CPC. Two pairs of right- and left-handed coil assemblies were alternately connected in series with flow tubes in such a way that the distal (non-gear) terminal of the first column assembly is connected to the proximal terminal (close to the center of rev-

olution) of the second column assembly and so forth. Four coil assemblies were symmetrically mounted on the rotary frame for balancing the centrifuge system as shown in Fig. 4. The total column capacity was 54 mL.

2.3. 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, USA). Dibasic potassium phosphate was obtained from Wako (Osaka, Japan). All other reagents were of reagent grade.

2.4. Preparation of aqueous–aqueous polymer phase systems 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 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 injecting 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-10ADVP, Shimadzu, Kyoto, Japan), while the column was rotated at a given revolution speed. The effluent from the column outlet was collected into test tubes at 0.8 mL/tube using a fraction collector (Model SF-160, Advantec, Tokyo, Japan).

2.6. Analysis of CCC fractions

Each collected protein fraction was diluted with 2 mL of distilled water and the absorbance was measured at 280 and 540 nm with a spectrophotometer (Model UV-1600, Shimadzu).

2.7. 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 (Rs).

3. Results and discussion

3.1. Design and fabrication of new small-scale X-axis CPC

Our new small-scale X-axis CPC was designed according to the following five concepts: (1) improvement of partition efficiencies; (2) protection of flow tubes during high speed revolution; (3) stability of column rotation; (4) adjustments to suitable operating conditions for various kinds of samples; and (5) simplification of operation and maintenance of the apparatus. The column temperature should also be maintained near the ambient level especially for labile biological samples.

Therefore, the fundamental plan for fabrication of the apparatus was proposed as follows: (1) in order to achieve the stability of the centrifuge system during the high-speed rotation, four separation columns of almost the same weight are symmetrically mounted on the rotary frame; (2) the twist-free single-line layout of flow tubes facilitates protection of flow tubes from mechanical damage during high-speed rotation; and (3) in order to ease mounting the column assemblies, the column holders are driven by the combination of a pair of miter gears and a set of spur gears without pulleys and toothed belts that were used in the original model.

Fig. 1 illustrates principle of the new design of a twist-free single-line flow tube layout. The twist caused by the revolution

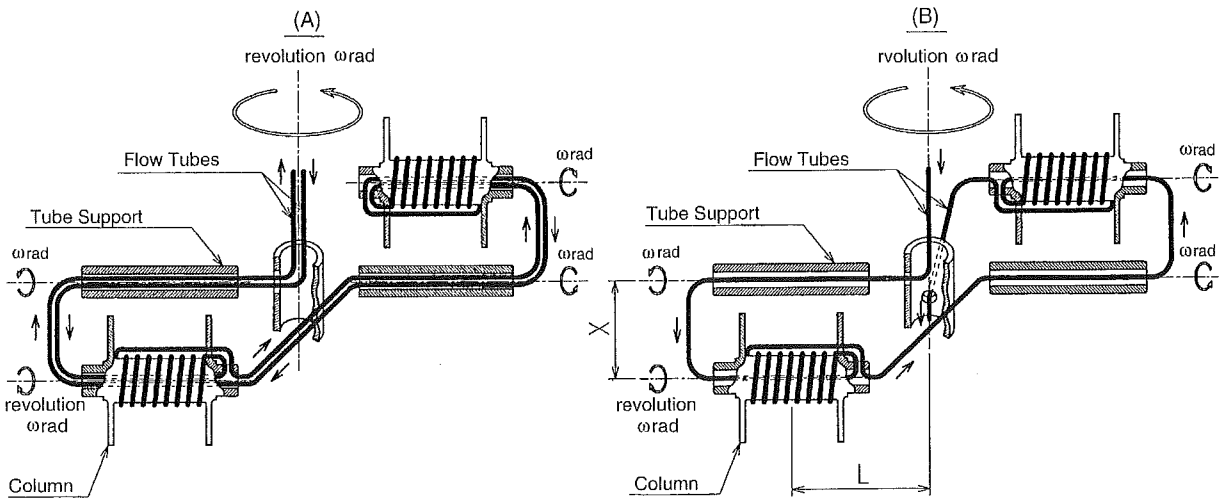

Fig. 2. Flow tube distributions in the original model of X-axis CPC: (A) traditional distribution and (B) new design of a single-line distribution. Counter-clockwise revolution is shown.

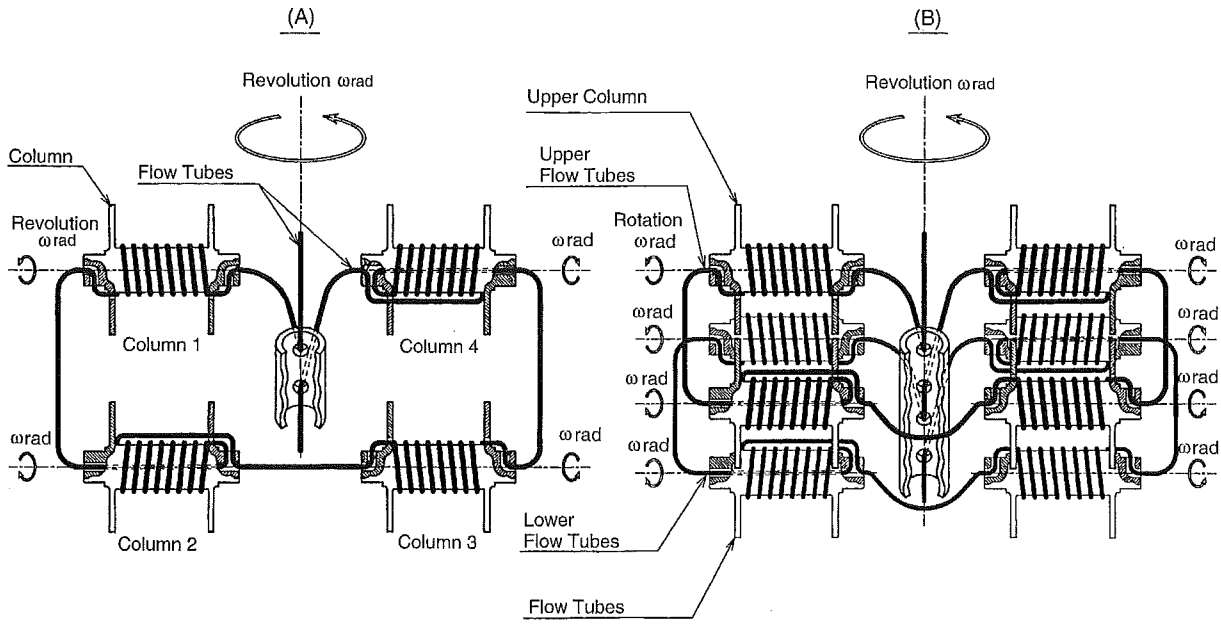

Fig. 3. Flow tube distributions applied to the new small-scale *X*-axis CPC: (A) single-line distribution for four column assemblies used in the present studies and (B) single-line distribution for several more column assemblies.

is canceled out by the twist caused by synchronous column rotation. The flow tube enters column 1 from the upper side of the apparatus (Fig. 1, top) and the rotary frame revolves counter-clockwise viewed from the top to avoid twisting the flow tube at point 1. Twisting the interconnection flow tubes is prevented by synchronous counter-rotation of the neighboring columns. Since the outlet flow tube from column 4 rotates in an opposite direction to column 1, the outlet flow tube is led downward to

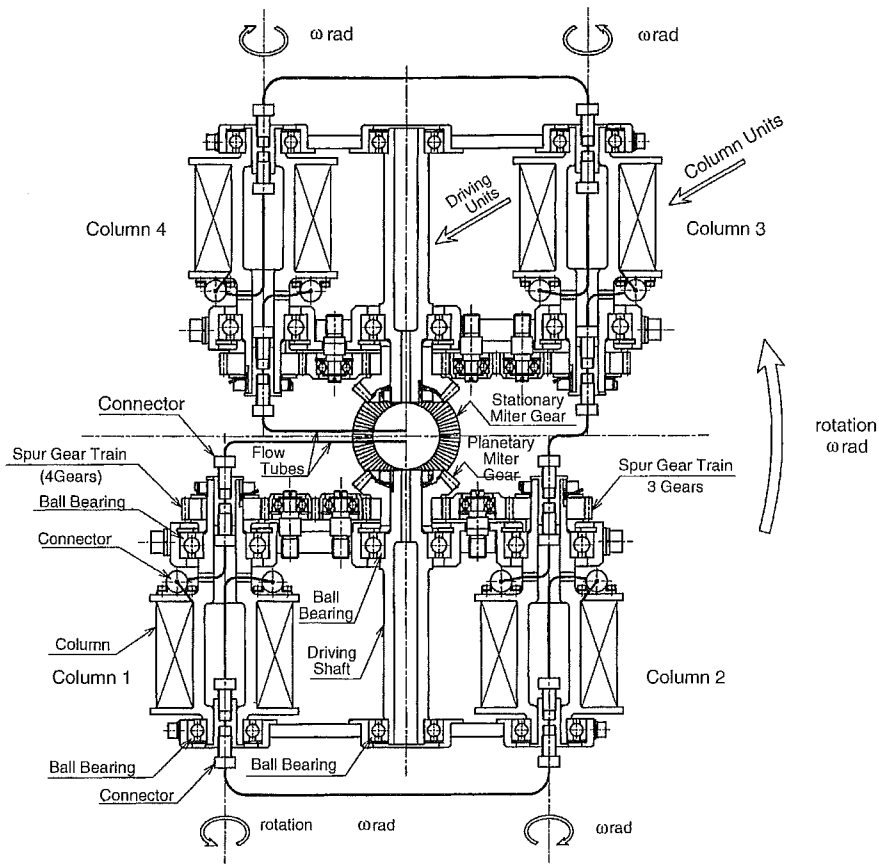

Fig. 4. Schematic drawing of the horizontal cross-sectional view through the center of columns in the small-scale *X*-axis CPC.

avoid twisting the flow tube at point 3 (Fig. 1, bottom). When viewed by an observer outside the system, columns 1 and 4, and columns 2 and 3 seem to rotate in the same direction, respectively. However, looking at the proximal side of each column from the point of revolution central axis results in that columns 1 and 3 rotate in one direction while columns 2 and 4 in the other direction to avoid the flow tube twisting. Viewing at the center of revolution gives that column 2 turns left while column 3 turns right. Therefore, the rotating direction of the column defines according to this point of view in the present studies.

Fig. 2A illustrates the flow tube distribution in the original model, and Fig. 2B the new flow tube distribution applied to the original model where a single flow tube runs between two columns. In this flow tube distribution, the first column connected by the flow tube entering from the upper side of the apparatus through the tube support corresponds to column 2 illustrated in Figs. 1 and 3A, and the second column connected by the flow tube between the proximal terminal and the distal terminal of the first column corresponds to column 4 rotating in the same direction of column 2. The tube supports in Fig. 2B also correspond to columns 1 and 3, respectively, both rotating in the opposite direction of columns 2 and 4.

Fig. 3A shows the flow tube distribution applied to the present apparatus. According to our proposed principle, it is possible to connect several more columns with single flow tubes as illustrated in Fig. 3B. Fig. 4 illustrates the schematic drawing of the horizontal cross-sectional view through the center of columns. The symmetrical distribution of 4 column assemblies with a single line of flow tubes provides stability of the system, lower noise and durability of the flow tube at high-speed revolution.

Fig. 5 shows the overview of the present apparatus without the cover case (the upper photograph), and the drive units (the lower photograph). The motor drives the rotary frame via a pair

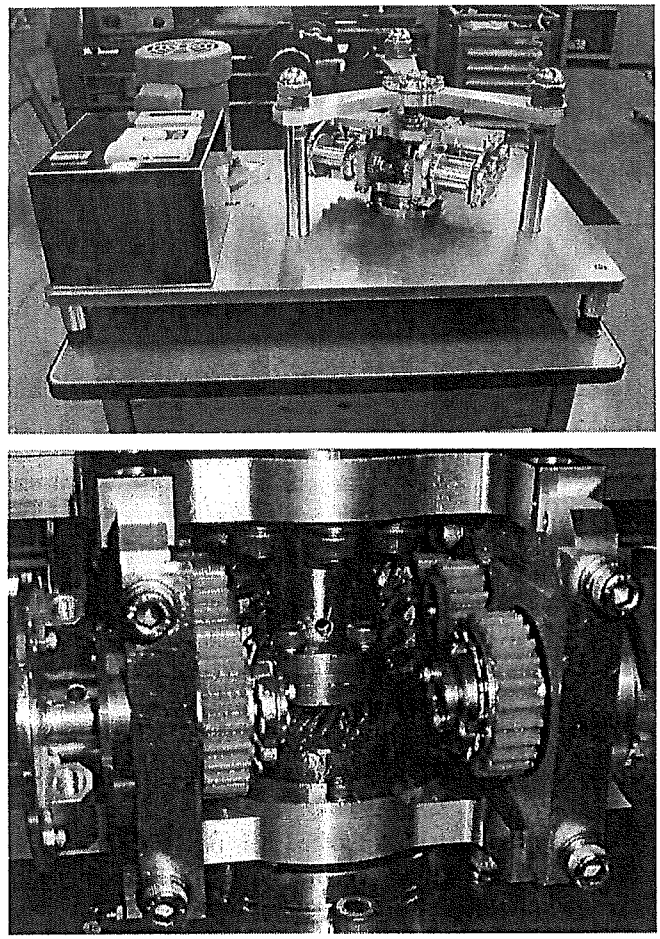

Fig. 5. Overview of the present *X*-axis CPC without the cover case (the upper photograph) and the drive units (the lower photograph).

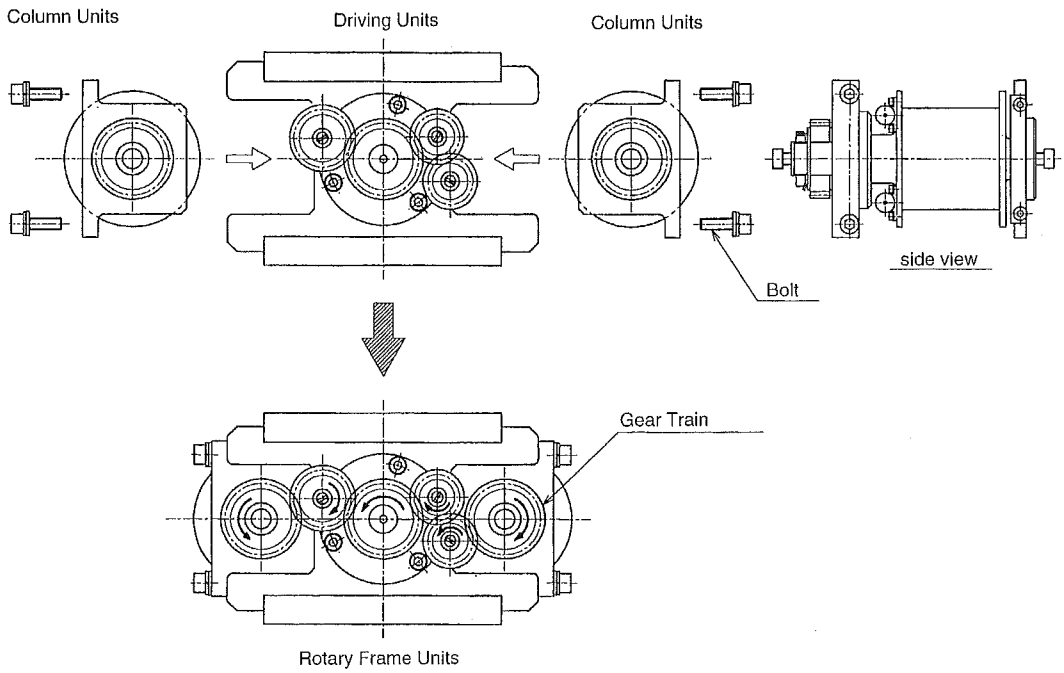

Fig. 6. Schematic drawing of the drive units in the small-scale *X*-axis CPC without the toothed belt.

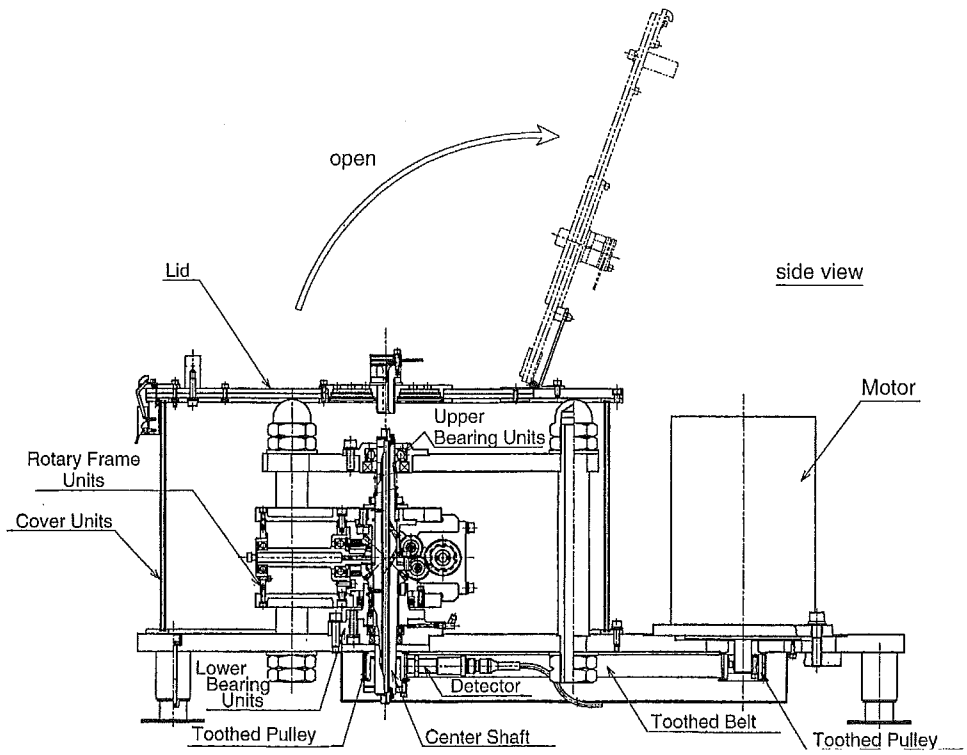

Fig. 7. Side view of the new small-scale *X*-axis CPC.

of pulleys coupled with a toothed belt (see Fig. 7), and this motion is conveyed to the column holder by the combination of several miter gears without the use of two toothed belts. In this way, the separation column is easily mounted on the rotary frame as mentioned earlier. The schematic drawing of the drive unit is shown in Fig. 6. The side view of the apparatus is also illustrated in Fig. 7.

In order to regulate the column temperature, the central portion of the ceiling of the centrifuge case is made by a thin metal plate with multiple perforations to maintain the column temperature near the ambient level during separation (Fig. 8).

Fig. 9 illustrates the horizontal cross-sectional view of the present apparatus. The dimensions of the bench-top model are no more than 80 cm in width and 55 cm in length. The pho-

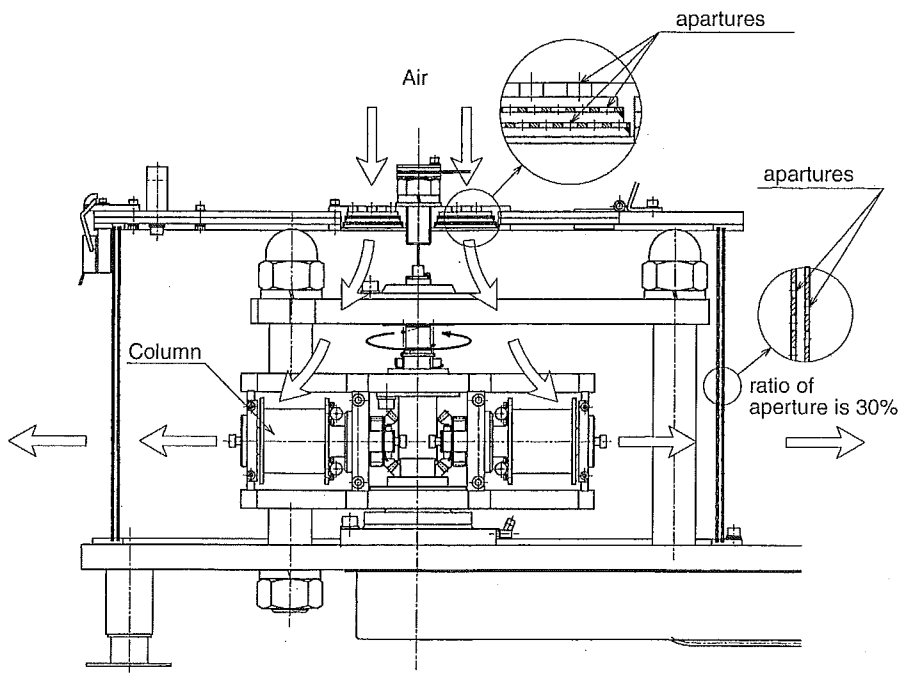

Fig. 8. Regulation of the column temperature using the cover case with multiple perforations in the central portion of the ceiling.

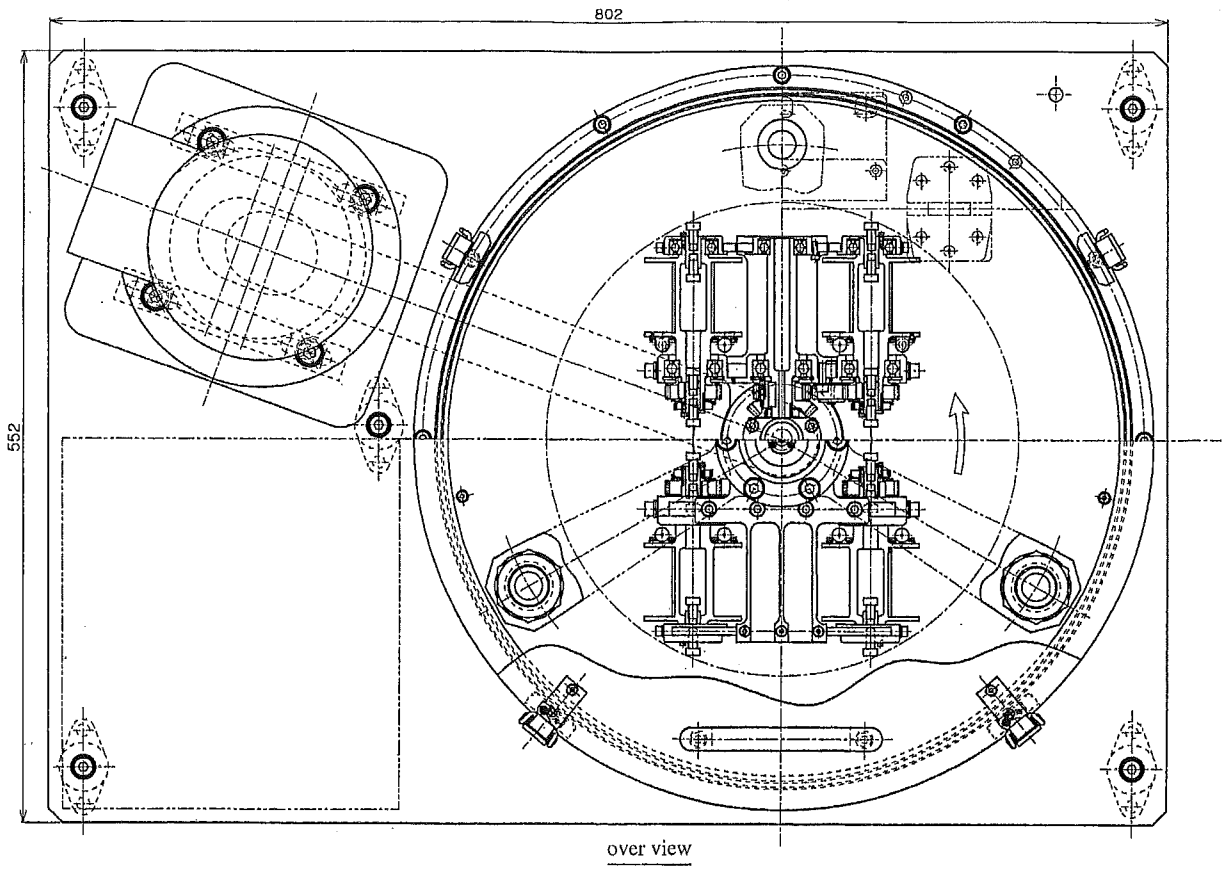

Fig. 9. Schematic drawing of the horizontal cross-sectional view of the present *X*-axis CPC.

tographs of the whole apparatus are also shown in Fig. 10. Table 1 summarizes various dimensions of the apparatus, the number of columns and their capacities, etc. compared between the original and present apparatus. The size and column volume are decreased to about 0.3 and 0.6 ratio, respectively, while the revolution speed and centrifugal force are increased by about 2.5 and 4 times those of the original model, respectively.

3.2. *Partition efficiency of proteins with aqueous–aqueous polymer phase systems*

Better separation using the *X*-axis CPC requires a series of experiments for searching the suitable combinations of the column configurations and the elution modes. Especially, the revolution direction of the column (clockwise or counter-clockwise) and the elution modes such as "head–tail" (head to tail or tail to head) and "inward–outward" (elute the mobile phase inwardly from the distal terminal of the fifth coiled layer of column 4 to the proximal terminal of the first coiled layer of column 1 in a series or outwardly in the reversed direction against the inward elution) affect the peak resolution for each column configuration of right- and left-handed coils.

The performance of the present apparatus was examined for the separation of proteins with an aqueous–aqueous polymer phase system. The separations were performed using a set of standard proteins including cytochrome c, myoglobin and lysozyme with a two-phase solvent system composed of 12.5% (w/w) PEG 1000 and 12.5% (w/w) dibasic potassium phosphate.

Table 1
Comparison of capabilities between original and present apparatuses

Subject	Original apparatus	Present apparatus	Ratio (present/original)
Revolution distance (mm)	408	241	0.59
Height at the center of the column (mm)	635	184	0.29
Column holder size (cm^3)[a]	955	283	0.30
The number of columns (unit)	2	4	2
Total column holder size (cm^3)[a]	1910	1130	0.59
Rotation speed (rpm)	800	2000	2.5
Centrifugal force by revolution (G)	146	537	3.7
Centrifugal force by rotation (average) (G)	35.7	134	3.8

[a] The space of the column holder to accommodate the multilayer coil.

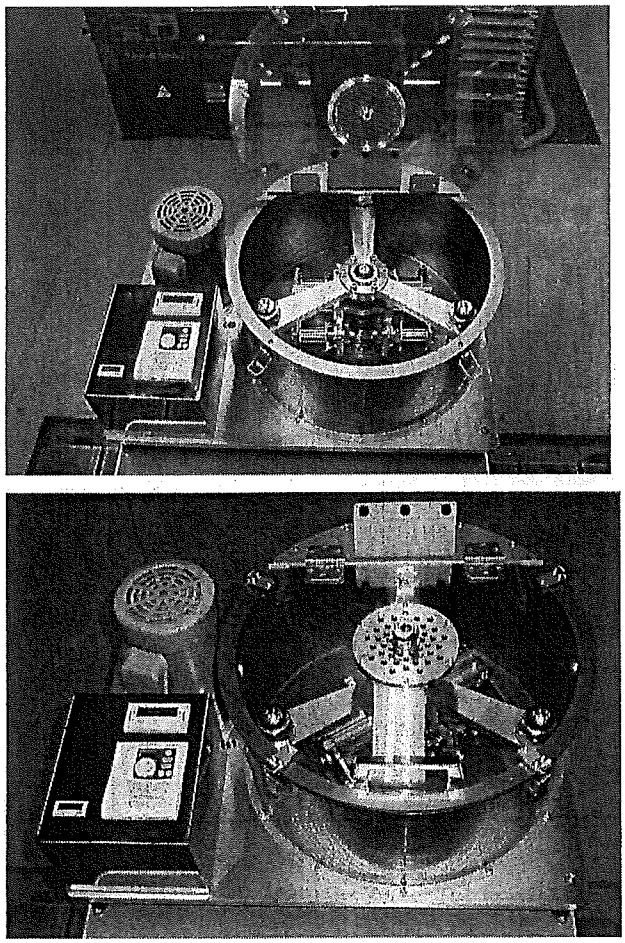

Fig. 10. Photographs of the whole new small-scale *X*-axis CPC with the cover case.

Successful CCC separation highly depends on the choice of the two-phase solvent system, which provides suitable partition coefficient (K) values for a set of analytes. In practical application, the K-value of the target protein is adjusted to less than $K=1$ so that the separation is completed in a short period of time. The measurement of the K-values using the simple test tube experiment [18] may be useful for predicting the retention time in CCC separation. In the present aqueous–aqueous polymer phase system, the K-value (C_U/C_L: the absorbance of the upper phase divided by that of the lower phase at 280 nm) of each standard protein was 0.02 for cytochrome c, 0.59 for myoglobin and 2.19 for lysozyme. Here, cytochrome c is useful as a marker since it elutes at the solvent front when the lower phase is used as the mobile phase, while it would retain in the column almost permanently when the upper phase is used as the mobile phase. Myoglobin (M.W. 17,800) and lysozyme (M.W. 14,400) can also be used as standard samples to evaluate the partition efficiency in terms of theoretical plate numbers and peak resolution, where they may not be resolved well by size-exclusion chromatography due to their similar molecular weights. In order to retain the stationary phase in the column, the lower phase is always eluted outwardly from the proximal terminal of the column and the upper phase inwardly from the distal terminal of the column.

Fig. 11 illustrates a set of CCC chromatograms obtained by eluting the lower phase under counter-clockwise revolution. The column combinations examined in this experiment were all left-handed multilayer coils (tail to head elution for columns 1 and 3, and head to tail elution for columns 2 and 4) shown in the top chromatograms and a combination of right-handed coils (column 1 and 3) and left-handed coils (column 2 and 4), and head to tail elution in all columns in the bottom chromatograms. As indicated in Fig. 1 and described above, columns 1 and 3 rotate in one direction while columns 2 and 4 rotate in the other direction. The increase of revolution speed improved the peak resolution in both columns, Table 2, while retention of stationary phase was reduced in the all-left-handed coils. The proper combination of right- and left-handed coils attained satisfactory retention of stationary phase to achieve sufficient peak resolution. These results suggest that in this solvent system the lower phase moves from the head toward the tail of the coiled column. As summarized in Table 2, the best separation was obtained at the revolution speed of 1000 rpm yielding resolution between the cytochrome c and myoglobin peaks of 1.6 and between myoglobin and lysozyme

Table 2
Analytical values computed from CCC chromatograms of proteins obtained by the lower phase mobile in the outward elution mode using the small-scale *X*-axis CPC

Column configuration and elution mode		Revolution (rpm)	Retention time (min)			Resolution factor (Rs)		Theoretical plates (N)	Stationary phase retention (%)
Column 1 and 3	Column 2 and 4		Cyt C	Myo	Lys	Cyt C/Myo	Myo/Lys		
Counter-clockwise revolution, Fig. 11									
Left-handed, T to H	Left-handed, H to T	800	99	118	153	1.2	1.1	393	13.9
		900	96	114	153	1.1	1.2	386	23.2
		1000	98	114	152	1.2	1.5	468	22.6
		1100	104	120	156	1.3	1.5	716	24.1
Right-handed, H to T	Left-handed, H to T	800	104	117	158	1.0	1.4	596	23.7
		900	102	118	160	1.2	1.5	580	24.1
		1000	118	140	184	1.6	1.5	846	30.7
		1100	96	114	164	1.4	1.7	537	33.3
Clockwise revolution, Fig. 13 left									
Left-handed, H to T	Right-handed, H to T	1000	94	112	164	1.3	1.9	526	28.7

Note: Cyt C, cytochrome c; Myo, myoglobin; Lys, lysozyme; H, head; T, tail. The theoretical plates were computed from the myoglobin peak of each chromatogram.

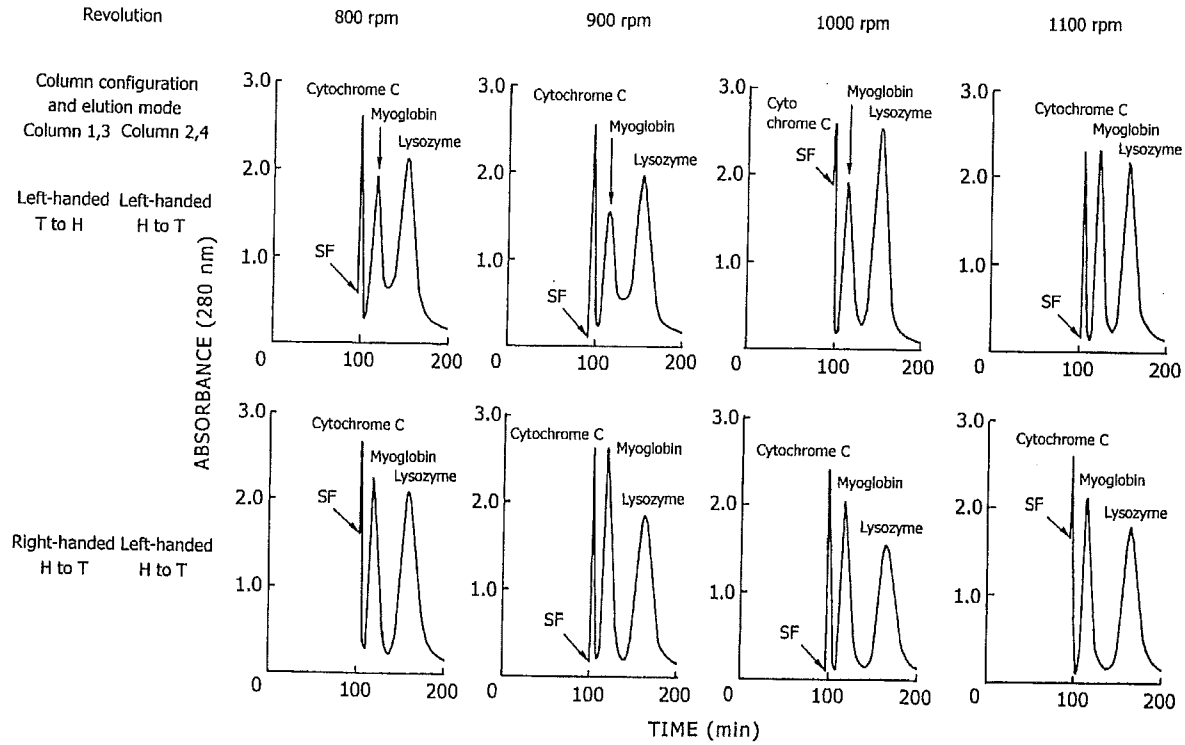

Fig. 11. CCC Chromatograms of proteins obtained by eluting the lower phase outwardly under counter-clockwise revolution. Experimental conditions: apparatus: small-scale *X*-axis CPC equipped with four multilayer coil assemblies, 1 mm ID and 54 mL total column capacity; sample: cytochrome c (2.5 mg), myoglobin (8 mg) and lysozyme (10 mg); solvent system: 12.5% (w/w) polyethylene glycol 1000–12.5% (w/w) dibasic potassium phosphate; mobile phase: lower phase; flow rate: 0.4 mL/min. Other conditions are described in the figure. See also Table 2. SF: solvent front.

peaks of 1.5. The theoretical plate number, *N*, was 846 as calculated from the myoglobin peak in the chromatogram.

When the upper mobile phase was eluted inwardly under counter-clockwise revolution, Fig. 12 and Table 3, the combination of right-handed columns 1 and 3 and left-handed columns 2 and 4 (tail to head elution for all columns, which is the reversed direction of lower phase mobile illustrated in the bottom chromatograms of Fig. 11) had low retention of stationary phase and insufficient protein separation as illustrated in the left chromatogram in Fig. 12. By changing the columns 1 and 3 from right- to left-handed (the elution mode is also changed from the tail toward the head to the head toward the tail while columns 2 and 4 are still kept in left-handed coils eluting from the tail toward the head inwardly), the peak resolution and stationary

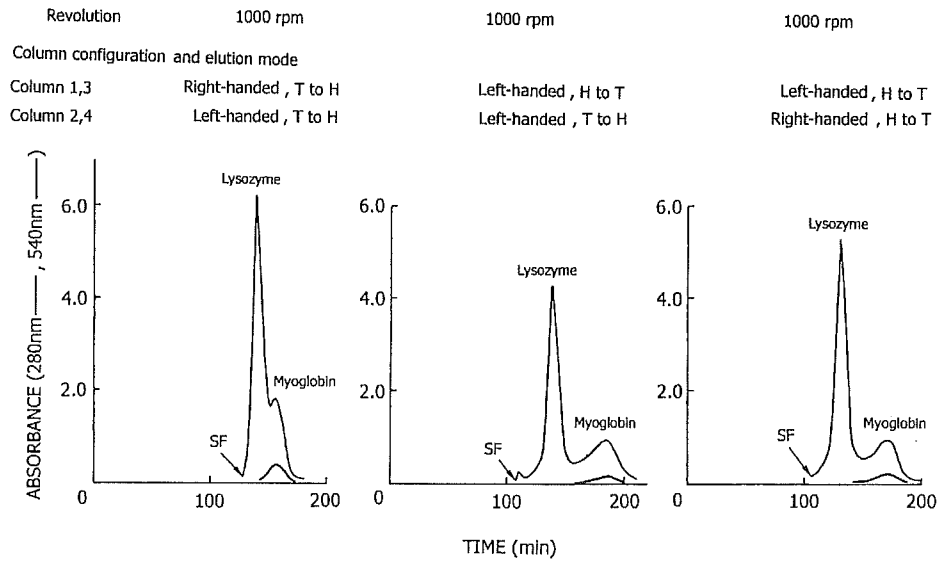

Fig. 12. CCC Chromatograms of proteins obtained by eluting the upper phase inwardly under counter-clockwise revolution. Experimental conditions: sample: lysozyme (10 mg) and myoglobin (8 mg); mobile phase: upper phase. Other conditions are described in Fig. 11 caption and in the figure. See also Table 3. SF: solvent front.

Table 3
Analytical values computed from CCC chromatograms of proteins obtained by the upper phase mobile in the inward elution mode using the small-scale *X*-axis CPC

Column configuration and elution mode		Revolution (rpm)	Retention time (min)		Resolution factor (Rs)	Theoretical plates (*N*)	Stationary phase retention (%)
Column 1 and 3	Column 2 and 4		Lys	Myo	Lys/Myo		
Counter-clockwise revolution, Fig. 12							
Right-handed, T to H	Left-handed, T to H	1000	140	155	0.6	968	1.9
Left-handed, H to T	Left-handed, T to H	1000	139	184	1.1	802	18.1
Left-handed, H to T	Right-handed, H to T	1000	121	169	1.6	633	22.6
Clockwise revolution, Fig. 13 right							
Right-handed, H to T	Left-handed, H to T	1000	135	168	1.3	903	15.1

Note: Lys, lysozyme; Myo, myoglobin; H, head; T, tail. The theoretical plates were computed from the lysozyme peak of each chromatogram.

phase retention were remarkably improved as illustrated in the central chromatogram of Fig. 12 and summarized in Table 3. Better protein separation with mobile the upper phase was achieved by the combination of left-handed (column 1 and 3) and right-handed (column 2 and 4) coils (head to tail elution for all columns) as shown in the right chromatogram in Fig. 12. As summarized in Table 3, the resolution between lysozyme and myoglobin peaks was 1.6 with the stationary phase retention at 22.6%. In short, the best partition efficiencies were obtained by the combination of right- and left-handed columns, i.e., right-handed columns 1 and 3 and left-handed columns 2 and 4 if the lower phase is the mobile phase with outward elution (all columns eluted from the head toward the tail), and left-handed columns 1 and 3 and right-handed columns 2 and 4 if the upper phase is the mobile phase with inward elution (all columns eluted from the head toward the tail) under counter-clockwise revolution.

Fig. 13 illustrates the CCC chromatograms obtained by clockwise revolution and both phases used as the mobile phase. Numerical parameters are summarized in Tables 2 and 3. The combination of left-handed (column 1 and 3) and right-handed (column 2 and 4) coils (right illustration), which produced sufficient peak resolution with mobile upper phase under counter-clockwise revolution as illustrated in the right chromatogram of Fig. 12, also yielded satisfactory separation of each protein with mobile lower phase. Moreover, the combination of right-handed (column 1 and 3) and left-handed (column 2 and 4) coils (left illustration) (all columns in the head to tail elution mode), which gave low retention of stationary phase with counter-clockwise revolution and tail to head elution mode as illustrated in the left chromatogram of Fig. 12, also accomplished a satisfactory separation with the upper phase mobile.

Table 4 summarizes the experimental conditions for performing better protein separations including the column configuration, head–tail and inward–outward elution modes. In practice, the columns may be arranged in such a way that columns 1 and 3 are right-handed and 2 and 4 are left-handed as shown in Fig. 3, left. When the lower phase is used as the mobile phase, it should be pumped through the upper flow tube while the apparatus is

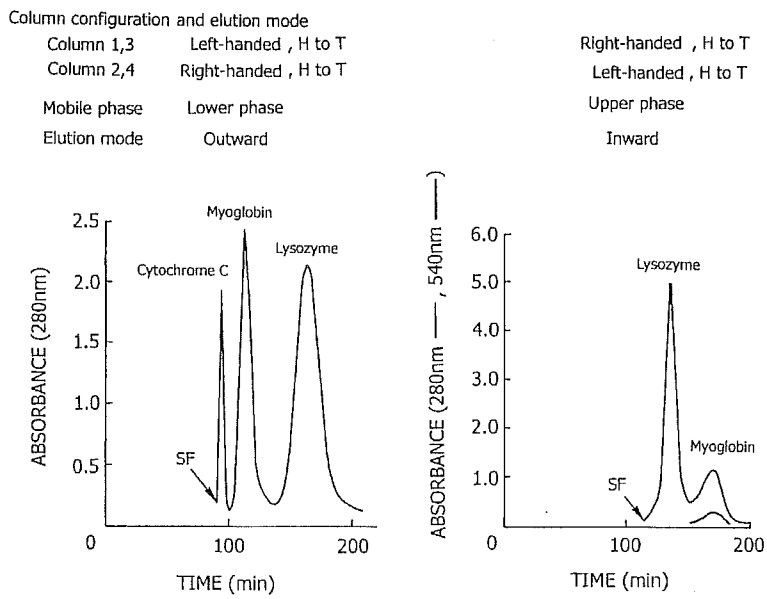

Fig. 13. CCC Chromatograms of proteins obtained by the new small-scale *X*-axis CPC under clockwise revolution. Experimental conditions are described in Fig. 11 caption for the lower phase mobile, Fig. 12 caption for the upper phase mobile and in the figure. See also Tables 2 and 3. SF: solvent front.

Table 4
Experimental conditions for performing better CCC separation using the present small-scale *X*-axis CPC

Column configuration		Mobile phase	Inward–outward elution mode	Head to tail elution mode	Revolution direction	Reference
Column 1 and 3	Column 2 and 4					
Right-handed	Left-handed	Lower phase	Outward	H to T (all columns)	Counter-clockwise	Fig. 11
		Upper phase	Inward	H to T (all columns)	Clockwise	Fig. 13, right
Left-handed	Right-handed	Lower phase	Outward	H to T (all columns)	Clockwise	Fig. 13, left
		Upper phase	Inward	H to T (all columns)	Counter-clockwise	Fig. 12

Note: H, head; T, tail.

rotated counter-clockwise (all columns in the head to tail elution mode), whereas an upper mobile phase should be pumped through the lower flow tube (by switching the inlet and the outlet) while the column is rotated clockwise so that all columns are eluted again in the head to tail mode.

4. Conclusion

The overall results indicate that the combination of right- and left-handed coils gave good partition efficiencies regardless of the choice of the mobile phase. The best results are obtained by eluting the lower mobile phase outwardly through the column in the head to tail elution mode and also by eluting the upper phase inwardly through the column in the head to tail elution mode. The overall results of our studies suggest that the retention of the stationary phase in the present apparatus is affected by both "head–tail" and "inward–outward" elution modes. This apparatus would be useful for the separation of various bioactive compounds with polymer phase systems.

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