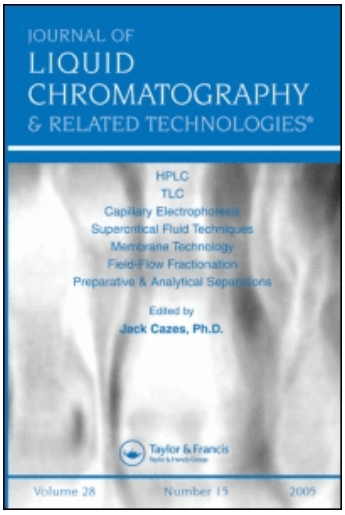

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Partition Efficiency of Newly Designed Locular Multilayer Coil for Countercurrent Chromatographic Separation of Proteins Using Small-Scale Cross-Axis Coil Planet Centrifuge with Aqueous-Aqueous Polymer Phase Systems

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Abstract: Countercurrent chromatographic performance of the locular multilayer coil separation column newly designed in our laboratory, was evaluated in terms of theoretical plate number, peak resolution, and retention of the stationary phase in protein separation with an aqueous polymer phase system, using the small-scale cross-axis coil planet centrifuge (X-axis CPC) fabricated in our laboratory. The locular column was made from 1.0 mm I.D., 2.0 mm O.D., or 1.5 mm I.D., 2.5 mm O.D. PTFE tubing, compressed with a pair of hemostat at 2 or 4 cm intervals. The protein separation was performed using a set of stable proteins including cytochrome C, myoglobin, and lysozyme with the 12.5% (w/w) polyethylene glycol 1000 and 12.5% (w/w) dibasic potassium phosphate system under 1000 rpm of column revolution. The 1.5 mm I.D., 2.5 mm O.D. locular tubing compressed at 2 cm intervals yielded better partition efficiencies than the non-clamped tubing, using both lower and upper mobile phases with satisfactory retention of the stationary phase. The overall results suggest that the newly designed locular multilayer coil is useful to the preparative separation

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of proteins with aqueous-aqueous polymer phase system using our small scale X-axis CPC.

Keywords: Aqueous-aqueous polymer phase system, Countercurrent chromatography, Cross-axis coil planet centrifuge, Locular multilayer coil, Protein, Separation

INTRODUCTION

The cross-axis coil planet centrifuge (X-axis CPC) has been used for performing countercurrent chromatographic (CCC) separation of macromolecules with aqueous-aqueous polymer phase systems.[1–4] This apparatus undergoes 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 planetary motion provides the use of low interfacial tension two-phase solvent systems, such as aqueous-aqueous polymer phase systems, which are generally not efficiently applied to type-J multilayer CPC. Our previous studies demonstrated that the floor model we have built with a pair of separation columns was useful for separation of proteins with aqueous-aqueous polymer phase systems.[7] Recently, a new small scale X-axis CPC has been designed and fabricated to improve the partition efficiency to promote the utility of CCC apparatus.[8] A series of experiments revealed that the apparatus was very useful for separation of proteins using aqueous-aqueous polymer phase systems. This small-scale X-axis CPC has a distinctive feature, such that four separation columns of similar weight are mounted symmetrically around the rotary frame to achieve stable balancing of the centrifuge under a high revolution speed. Our recent studies demonstrated that the improved apparatus was also useful for the purification of ribonuclease (RNase) from the extract of bullfrog egg, using an aqueous-aqueous polymer phase system without loss of the native activity.[9]

Partition efficiency in this X-axis CPC varies according to the type of the coiled column including multilayer coil,[8–10] eccentric coil,[7,11,12] toroidal coil,[11,12] spiral coil,[13] and so forth. Our previous studies revealed that among these coiled columns the eccentric coil is most useful for protein separation in the analytical scale and the multilayer coil for the preparative scale separations. In order to achieve sufficient partitioning of the solutes during the separation, the two phases require numerous times of mixing and settling in the coiled column. The present paper describes the newly designed locular multilayer coil assembly and its performance in protein separation using the small-scale X-axis CPC with an aqueous-aqueous solvent system composed of polyethylene glycol (PEG) 1000 and dibasic potassium phosphate.

EXPERIMENTAL

Apparatus

The small-scale X-axis CPC employed in the present study was constructed at the Machining Technology Center of Nihon University, Chiba, Japan. The design and fabrication of the apparatus and the column configuration were previously described in detail.[8,9]

Preparation of Locular Tubing

The newly designed locular tubing was prepared using a piece of commercial 1.0 mm I.D., 2.0 mm O.D. or 1.5 mm I.D., 2.5 mm O.D. PTFE (polytetrafluoroethylene) tubing (Flon Kogyo, Tokyo, Japan) by compressing with a pair of hemostats at 2 or 4 cm intervals. Figure 1 illustrates the schematic drawing of the locular tubing.

Preparation of Coiled Column

Each multilayer coil was prepared by tightly winding a piece of PTFE tubing 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 side, which is close to the center of revolution. After each coil layer was

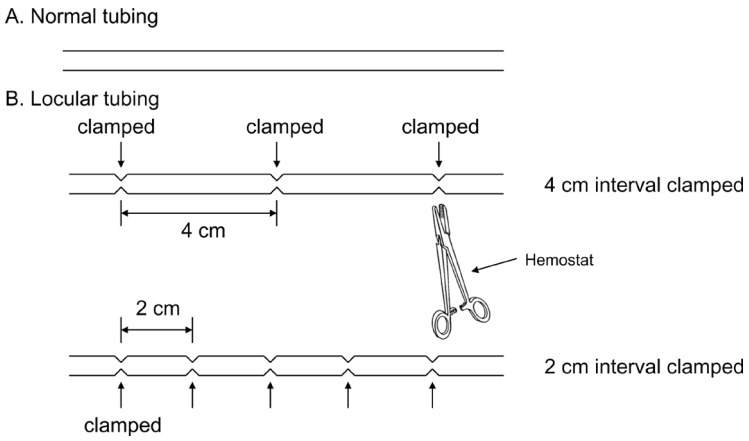

Figure 1. Schematic illustration of the newly designed locular tubing.

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 columns of left handed coils were subjected to the forward rotation; and right handed coils, the backward rotation. 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, which is close to the center of revolution, of the second column assembly, etc. Four coil assemblies were symmetrically mounted on the rotary frame for balancing the centrifuge system.

Reagents

Polyethylene glycol (PEG) 1000 (MW 1,000), cytochrome C (horse heart) (MW 12,384), myoglobin (horse skeletal muscle) (MW 17,800), and lysozyme (chicken egg) (MW 13,680) were purchased from Sigma (St. Louis, MO, USA). Dibasic potassium phosphate was obtained from Wako (Osaka, Japan). All other reagents were of reagent grade.

Preparation of Aqueous-Aqueous Polymer Phase Systems and Sample Solutions

A two-phase polymer system used in the present studies 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 formed.

The sample solutions were prepared by dissolving a set of standard proteins 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 injection of the sample solution through the flow tube leading to the head of CCC column by a syringe. 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 1000 rpm of revolution speed. The effluent from the column outlet was collected into test tubes using a fraction collector (Model CHF100AA, Advantec, Tokyo, Japan).

Analysis of CCC Fractions

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

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) each using the conventional formula.

RESULTS AND DISCUSSION

The partition efficiency for the new locular multilayer coiled column was evaluated by protein separation using the small-scale X-axis CPC with an aqueous-aqueous polymer phase system composed of 12.5% (w/w) PEG 1000 and 12.5% (w/w) dibasic potassium phosphate. Figures 2 and 3 illustrate the CCC separations of a set of three stable proteins including cytochrome C, myoglobin, and lysozyme, obtained by multilayer coils made from 1.0 mm I.D., 2.0 mm O.D. PTFE tubing (Figure 2) and 1.5 mm I.D. PTFE tubing (Figure 3), using the lower phase as a mobile phase. Table 1 summarizes the analytical data calculated from these chromatograms. In the 1.0 mm I.D., 2.0 mm O.D. PTFE tubing, the peak resolution and theoretical plate number are quite similar between the normal and the locular tubing compressed at 2 cm intervals, but in the locular tubing compressed at 4 cm intervals, the theoretical plate number is substantially increased without significant change in the retention of the stationary phase. At the 1.5 mm I.D., 2.5 mm O.D. tubing, the theoretical number calculated from the myoglobin peak becomes improved with the number of compressed sites along the tubing, while the peak resolution and stationary phase retention are kept almost constant. The overall result indicates that higher partition efficiency was obtained in the locular tubing than in the normal tubing, and the effect

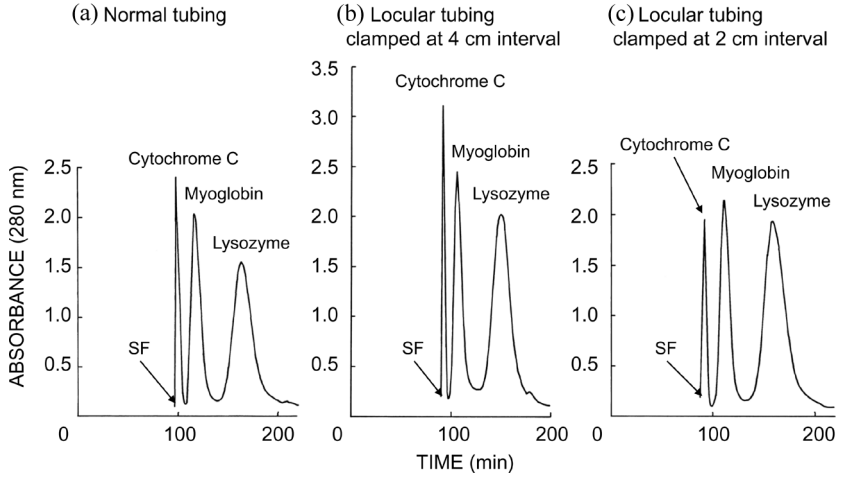

Figure 2. CCC separations of proteins obtained by 1.0 mm I.D., 2.0 mm O.D. PTFE locular multilayer coiled columns with a lower mobile phase. Experimental conditions: apparatus: small-scale X-axis CPC with three multilayer coil assemblies; total column capacity: (a) 54.0 mL, (b) 53.0 mL, and (c) 52.0 mL; 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 (outward elution); flow rate: 0.4 mL/min; revolution: 1000 rpm (counterclockwise). SF = solvent front.

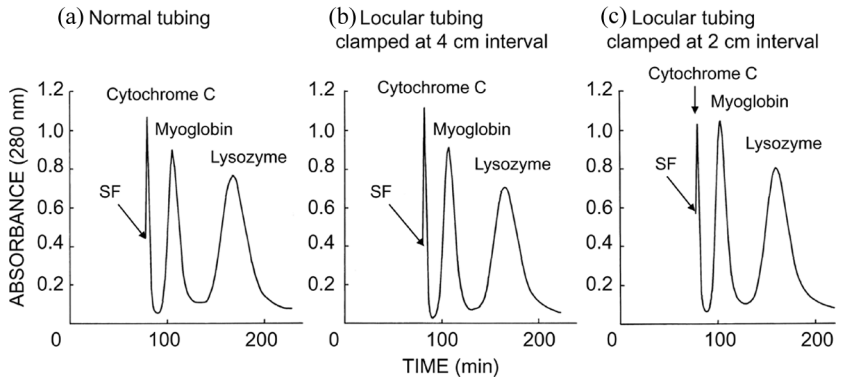

Figure 3. CCC separations of proteins obtained by locular multilayer coiled columns of 1.5 mm I.D., 2.5 mm O.D. PTFE tubing with a lower mobile phase. Experimental conditions: total column capacity: (a) 102.0 mL, (b) 98.0 mL, and (c) 97.0 mL; flow rate: 0.8 mL/min. Other experimental conditions are same as those described in Figure 1 caption. SF = solvent front.

Table 1. Analytical values obtained by CCC separations of proteins using two different diameter tubing for the multilayer coiled columns with lower phase mobile

Tubing	Elution volume (mL)			Peak resolution (Rs)		Theoretical plate number (N)	Theoretical plate number per column capacity (N/mL)	Stationary phase retention (%)
	Cyt C	Myo	Lys	Cyt C/Myo	Myo/Lys			
1.0 mm ID, 2.0 mm OD								
Normal	39.2	46.6	65.4	1.4	1.6	588	10.9	30.3
Locular (4 cm interval clamped)	36.8	42.4	60.0	1.4	1.8	867	16.4	32.1
Locular (2 cm interval clamped)	36.8	44.5	63.8	1.4	1.7	560	10.8	33.8
1.5 mm ID, 2.5 mm OD								
Normal	64.0	85.1	133.8	1.7	1.5	325	3.2	40.4
Locular (4 cm interval clamped)	65.6	85.8	131.5	1.7	1.6	330	3.4	36.3
Locular (2 cm interval clamped)	64.0	82.6	128.0	1.6	1.6	352	3.6	37.3

Abbreviations: Cyt C = cytochrome C; Myo = myoglobin; Lys = lysozyme. The average K values (C_U/C_L) for each protein were 0.09 for Cyt C, 0.56 for Myo and 1.73 for Lys, respectively.

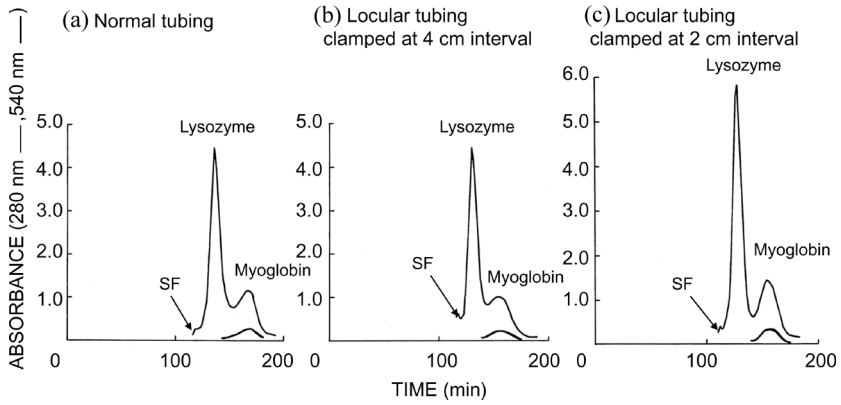

Figure 4. CCC separations of proteins obtained from the locular multilayer coiled columns of 1.0 mm I.D., 2.0 mm O.D. PTFE tubing with an upper mobile phase. Experimental conditions: sample: myoglobin (8 mg) and lysozyme (10 mg); mobile phase: upper phase (inward elution); revolution: 1000 rpm (clockwise). Other experimental conditions are same as those described in Figure 1 caption. SF = solvent front.

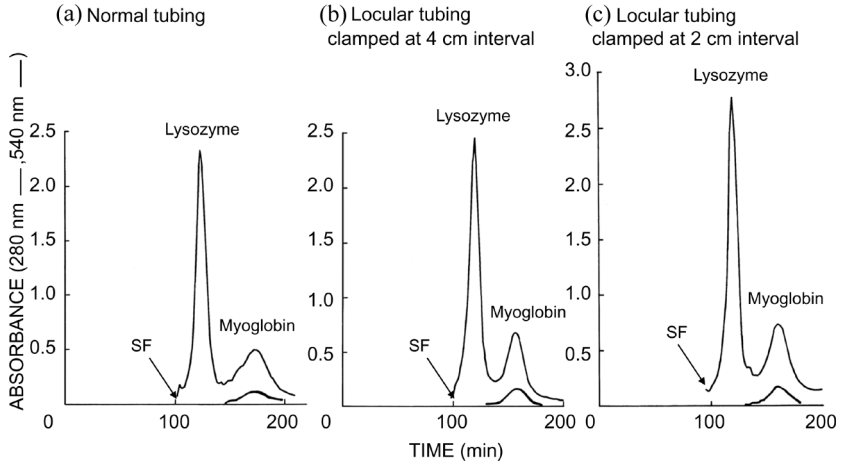

Figure 5. CCC separations of proteins obtained from the locular multilayer coiled columns with 1.5 mm I.D., 2.5 mm O.D. PTFE tubing with upper phase mobile. Experimental conditions: total column capacity: (a) 102.0 mL, (b) 98.0 mL, and (C) 97.0 mL; sample: myoglobin (8 mg) and lysozyme (10 mg); mobile phase: upper phase (inward elution); revolution: 1000 rpm (clockwise). Other experimental conditions are same as those described in Figure 1 caption. SF = solvent front.

Table 2. Analytical values obtained by CCC separations of proteins using two different diameter tubing for the multilayer coiled columns with upper phase mobile

Tubing	Elution volume (mL)		Peak resolution (Rs)	Theoretical plate number (N)	Theoretical plate number per column capacity (N/mL)	Stationary phase retention (%)
	Lys	Myo	Lys/Myo			
1.0 mm ID, 2.0 mm OD						
Normal	54.4	66.9	1.1	803	14.9	15.6
Locular (4 cm interval clamped)	52.0	61.6	0.9	835	15.8	14.0
Locular (2 cm interval clamped)	50.9	61.9	1.3	1011	19.4	16.9
1.5 mm ID, 2.5 mm OD						
Normal	99.2	138.2	1.4	667	6.5	23.1
Locular (4 cm interval clamped)	96.0	125.4	1.4	600	6.1	21.6
Locular (2 cm interval clamped)	96.0	128.0	1.5	744	7.7	22.5

Abbreviations: Lys = lysozyme; Myo = myoglobin. The average K values (C_L/C_U) for each protein were 0.92 for Lys and 2.41 for Myo, respectively.

of compression was clearly observed in the 1.5 mm I.D., 2.5 mm O.D. locular tubing, more than in the 1.0 mm I.D., 2.0 mm O.D. locular tubing.

Figures 4 and 5 illustrate the CCC separations of a set of two stable proteins, lysozyme and myoglobin, obtained by multilayer coils made from the 1.0 mm I.D., 2.0 mm O.D. tubing (Figure 4) and the 1.5 mm I.D., 2.5 mm O.D. tubing (Figure 5), using the upper phase as a mobile phase. Table 2 summarizes the analytical data calculated from these chromatograms. Both of the 1.0 mm I.D., 2.0 mm O.D. and the 1.5 mm I.D., 2.5 mm O.D. locular tubing, compressed at 2 cm intervals, produced the best separation in the present experiments. These results suggest that higher partition efficiency was obtained by the 2 cm interval clamped locular tubing more than the normal tubing with the upper phase mobile.

CONCLUSION

The newly designed locular multilayer coiled column mounted on our small-scale X-axis CPC performed better partition efficiency on protein separation with an aqueous-aqueous polymer phase system. In both elution modes, by using either phase as the mobile phase, better partition efficiencies were obtained in the 2 cm interval clamped locular tubing than the normal tubing. The effect of clamping was more significantly observed in the 1.5 mm I.D., 2.5 mm O.D. locular tubing than in the 1.0 mm I.D., 2.0 mm O.D. locular tubing. These results suggest that the locular tubing is useful for the efficient separation of proteins using aqueous-aqueous polymer phase systems at the preparative level.

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