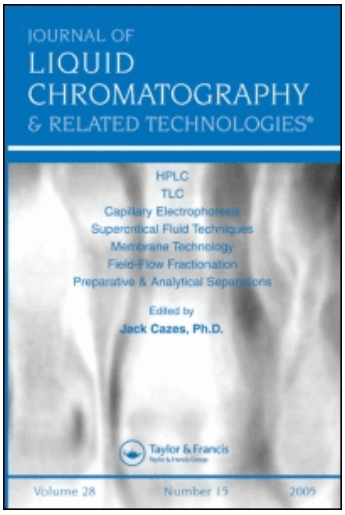

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PROTEIN SEPARATION BY CROSS-AXIS COIL PLANET CENTRIFUGE WITH TWO DIFFERENT TYPES OF COILED COLUMNS

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ABSTRACT

Proteins were separated using a cross-axis coil planet centrifuge equipped with two different types of coiled columns, i.e., toroidal and eccentric coil assemblies. An aqueous-aqueous polymer phase system composed of 12.5% (w/w) polyethylene glycol 1000 and 12.5% (w/w) dibasic potassium phosphate was used for the separation of cytochrome C, myoglobin and ovalbumin. The toroidal coil assemblies with 1 mm ID tubing yielded the partition efficiency of 170 theoretical plates at the myoglobin peak. A slightly higher efficiency was obtained from the eccentric coil assemblies with the same diameter. The highest partition efficiency of 450 theoretical plates was attained from the eccentric coil assemblies with 0.85 mm ID and 64 mL capacity.

INTRODUCTION

Countercurrent chromatography (CCC) is a continuous liquid-liquid partition method which operates without the use of solid support.[1,2] The stationary phase is retained in the column by the aid of gravity or centrifugal force; hence, the system eliminates all the complications arising from the use of a solid support. Among various CCC systems developed in the past, high-speed CCC has proven most useful since it provides advantages such as high peak resolution and short separation times, in addition to durability and stability of the instrument.[3,4] However, the apparatus does not retain the stationary phase well, if applied to aqueous-aqueous polymer systems characterized by low interfacial tension and high viscosity.

The cross-axis coil planet centrifuge (X-axis CPC) introduced to solve the above problem has a unique mode of planetary motion such that the column holder rotates about its horizontal axis while revolving around the vertical axis of the centrifuge.[5,6] This motion retains the stationary phase of aqueous-aqueous polymer phase systems, useful for partition of proteins.

In the past, the following three different types of separation columns were designed for the X-axis CPC: multilayer coil for preparative-scale separation, eccentric coil assembly, and toroidal coil for analytical-scale separation. Our previous studies demonstrated that the X-axis CPC equipped with either a multilayer coil or eccentric coil in the off-center position was useful for the separation of proteins with polyethylene glycol (PEG) - potassium phosphate solvent systems.[7-9]

The present paper describes the performance of the X-axis CPC equipped with the toroidal coil and eccentric coil assembly. A set of standard protein samples was used to evaluate the partition efficiency by varying ID and capacity of the column.

EXPERIMENTAL

Apparatus

The X-axis CPC employed in the present studies was constructed at the machine shop of Nihon University, Chiba, Japan. The design of the apparatus was previously described in detail[7,8] and a brief description is given here.

The apparatus produces a synchronous planetary motion of the column holder which rotates about its own horizontal axis and simultaneously revolves around the vertical axis of the apparatus, at the same angular velocity. A pair of column holders can be accomodated on the rotary frame, at two different positions, i.e., the off-center position with X = 10 cm and L = 15 cm (X is the distance between the holders at the central axis of the apparatus and L, the deviation of the holder position from mid portion of the rotary shaft). In the previous studies,[7,8] an eccentric coil assembly, mounted in the off-center position, produced efficient analytical separations of stable proteins. In the present studies, protein separation was performed in two different types of columns, the toroidal coil and eccentric coil assemblies, both mounted at the off-center position for comparative studies on their partition efficiencies.

Preparation of Two Coiled Columns

The toroidal coil assembly was prepared by winding a 1 mm ID PTFE (polytetrafluoroethylene) tubing (Flon Kogyo, Tokyo, Japan) onto a 125 cm long, 5 mm OD nylon pipe forming left handed coils. The coiled column was again coiled around the holder to form a left handed wound coil. A pair of identical coil assemblies was connected in series to obtain a total column capacity of 25 mL.

Each eccentric coil assembly was prepared by winding a 0.85 mm ID PTFE tubing (Zeus Industrial Products, Raritan, NJ, USA) onto 7.6 cm long, 5 mm OD nylon pipes making a series of tight left-handed coils. These coil units were arranged symmetrically around the holder hub of 7.6 cm OD in such a way that the axis of each coil unit is parallel to the holder axis. Two sets of coil assemblies were mounted on the rotary frame, one on each side, and serially connected with the flow tube. In order to investigate the effect of column ID on partition efficiency, columns with two different IDs (0.55 mm and 1.0 mm) were also employed.

Reagents

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

Preparation of the 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, allowing the mixture to settle into two clear layers before use.

CCC Separation of Proteins

In each separation, the column was first completely filled with the PEG-rich upper stationary phase. This was followed by injection of the sample solution through the sample port. Then, the apparatus was rotated at 800 rpm in a counterclockwise direction, while the phosphate-rich lower mobile phase was pumped into the column at a flow rate of 0.2 mL/min, which was mainly used throughout the present studies. The elution was performed in such a mode that the mobile phase flows against the action of the centrifugal force field to retain the lighter stationary phase in the column. The effluent from the outlet of the column was collected in test tubes (0.4 mL/tube) using a fraction collector (Model SF-200, Advantec Co., Tokyo, Japan).

Analysis of CCC Fractions

Each fraction was diluted with 2.5 mL of distilled water and the absorbance was measured at 280 nm using a spectrophotometer (Model UV-160, Shimadzu Corporation, Kyoto, Japan).

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). Both values are based on an assumption that each peak represents the distribution of a single component. Among the three standard proteins in the sample mixture, ovalbumin obtained from Sigma produced an extremely broad peak resulting in low values for both theoretical plate number and peak resolution. The previous studies revealed that this particular ovalbumin sample consisted of two components, as evidenced by the formation of two distinct bands in gel electrophoresis. The ovalbumin sample obtained

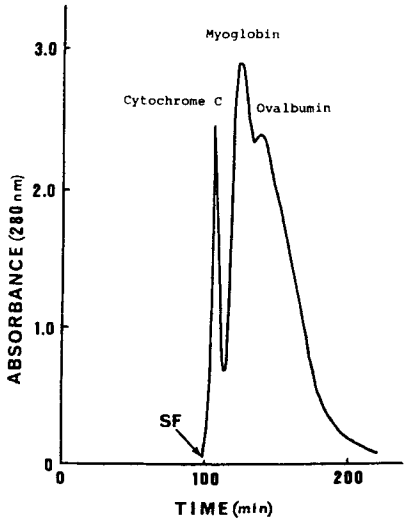

Figure 1. Chromatogram of the stable protein mixture obtained with the toroidal coil assemblies on the X-axis CPC. Experimental conditions were as follows: column: 1.0 mm ID with a total capacity of 25.0 mL; sample: cytochrome C (2.5 mg), myoglobin (8 mg), and ovalbumin (Wako) (30 mg); solvent system: 12.5% (w/w) PEG 1000 and 12.5% (w/w) dibasic potassium phosphate; mobile phase: lower phase; flow-rate: 0.2 mL/min; revolution: 800 rpm; SF = solvent front.

from Wako Chemicals formed a single band.[9] In the present studies, the ovalbumin sample purchased from Sigma was used to determine the optimum conditions of eccentric coil assemblies in order to clarify the effect of bore size of the tubing on separation.

RESULTS AND DISCUSSION

CCC Separation of Proteins by Toroidal Coil Assemblies

Our previous studies revealed that both eccentric coil and mutilayer coil assemblies were useful in protein separation by using the X-axis CPC with an aqueous polymer phase system composed of 12.5% (w/w) PEG 1000 and 12.5% (w/w) dibasic potassium phosphate.[7] Among these, the eccentric coil assembly produced efficient separation of cytochrome C (2.5 mg), myoglobin (8 mg) and

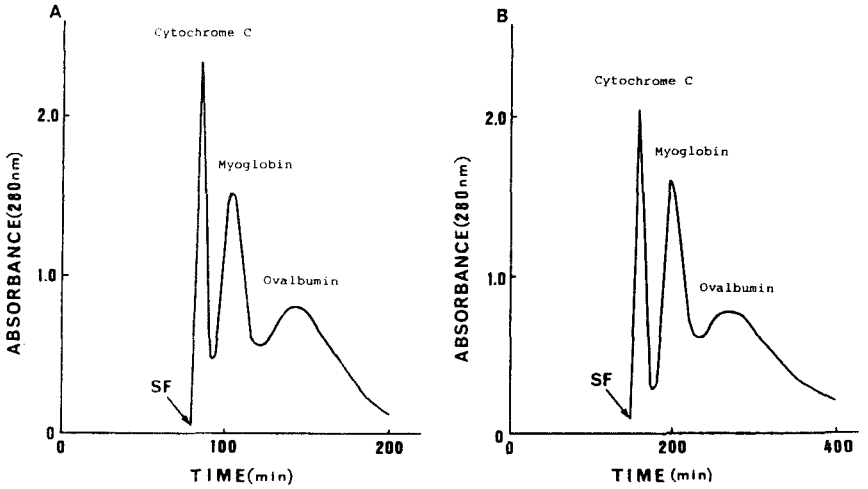

Figure 2. Chromatograms of stable protein mixture obtained from toroidal coil assemblies. Sample: cytochrome C (2.5 mg), myoglobin (4 mg), and ovalbumin (Wako) (15 mg); flow rate: 0.2 mL/min (A) and 0.1 mL/min (B). Other conditions were same as those described in the Fig. 1 caption. SF = solvent front.

ovalbumin (from Wako) (30 mg).[8] The partition efficiency computed from the myoglobin peak was 183 theoretical plates while the resolution between cytochrome C and myoglobin peaks was 1.1 and that between myoglobin and ovalbumin peaks, 0.9.

Fig. 1 illustrates the separation of cytochrome C (2.5 mg), myoglobin (8 mg) and ovalbumin (Wako) (30 mg) obtained by the X-axis CPC equipped with the toroidal coil assemblies. The sample size was equal to that used in the eccentric coil assemblies described above. Although the cytochrome C peak is fairly well resolved from the myoglobin peak, the myoglobin peak is only partially resolved from the ovalbumin peak suggesting that the toroidal coil is less efficient than the eccentric coil at this sample size.

Fig. 2 shows the separation of one-half of the same amount of sample, i.e., cytochrome C (2.5 mg), myoglobin (4 mg) and ovalbumin (Wako) (15 mg) by the toroidal coil assemblies. The reduced sample size substantially improved the peak resolution. At the flow rate of 0.2 mL/min (Fig. 2A), the partition efficiency computed from the myoglobin peak is 170 TP (theoretical plates), and the resolution between the cytochrome C and myoglobin peaks is 0.8, and that between the myoglobin and ovalbumin peaks, 0.6. When the flow rate was

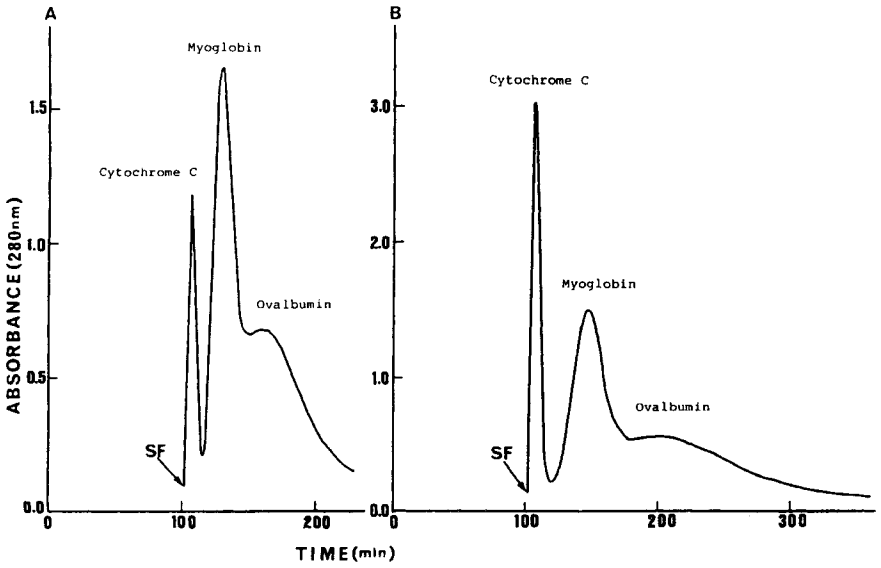

Figure 3. Chromatograms of stable protein mixture obtained from two different eccentric coil assemblies. Column: (A) 0.55 mm ID with a total capacity of 35.6 mL, (B) 1.0 mm ID with a total capacity of 32.4 mL; sample: (A) cytochrome C (1.2 mg), myoglobin (4 mg) and ovalbumin (Sigma) (15 mg), (B) cytochrome C (2.4 mg), myoglobin (8 mg) and ovalbumin (Sigma) (30 mg). Other conditions were same as those described in the Fig. 1 caption. SF = solvent front.

Table 1

Comparison of Partition Efficiencies Between Eccentric Coil and Toroidal Coil

	Total Turns of PTFE Tubing	Theoretical Plate (TP)	Theoretical Plate at 1 Turn of Tubing (TP)	Volume of Tubing at 1 Turn (mL)
Eccentric Coil*	1000	183	0.18	0.028
Toroidal Coil	1157	170	0.15	0.022

* The values for the eccentric coil were calculated from the results in our previous paper.[8]

reduced to 0.1 mL/min (Fig. 2B), the partition efficiency of the myoglobin peak became 153 TP while the peak resolution between the first (cytochrome C) and the second (myoglobin) peak was 0.8 and that between the second (myoglobin) and the third (ovalbumin) peaks, 0.5. In Table 1 the partition efficiencies between the eccentric and toroidal coil assemblies are compared. These results indicate that, at the reduced sample size, the toroidal coil gives an efficiency only slightly lower than that obtained by the eccentric coil assemblies.

Optimization of the Eccentric Coil Assemblies

In order to optimize the eccentric coil assemblies for separation of proteins, a series of experiments was conducted using three different IDs of tubing, 0.55 mm, 0.85 mm, and 1.0 mm. All experiments were performed with the same polymer phase system.

Fig. 3 shows separations of the standard protein mixture by the X-axis CPC equipped with a pair of eccentric coil assemblies of 0.55 mm (A) and 1.0 mm (B) IDs. Both chromatograms were obtained at 800 rpm at a flow rate of 0.2 mL/min with the column held in the off-center position. The total sample size was 20 mg for the 0.55 mm ID column and 40 mg for the 1.0 mm ID column. In both columns, the first and second peaks were well separated while the second and the third peaks were only partially resolved.

Comparison of these two chromatograms reveals that the wider column of 1.0 mm ID (B) yields slightly better resolution but lower theoretical plate numbers (131 TP) than the narrower column of 0.55 mm ID (180 TP) (A) measured with the second myoglobin peak. This may be due to better retention of the stationary phase in the wider bore column.

The chromatogram shown in Fig. 4A was obtained from a protein mixture loaded to the eccentric coil assemblies of 0.85 mm ID consisting of 32 coil units with a total capacity of 35.4 mL. The separation was performed under the identical conditions applied to the earlier studies described in Fig. 3, except that the sample size was increased to 60 mg. The first and the second peaks are well separated and the theoretical plate number measured from the second myoglobin peak is 256, which is substantially higher than those obtained from other eccentric coil assemblies with wider ID.

When the sample size was reduced to 40 mg and the length of the coil increased to 68 units with a total capacity of 64.5 mL, the separation was improved as shown in Fig. 4B where the third peak revealed a characteristic

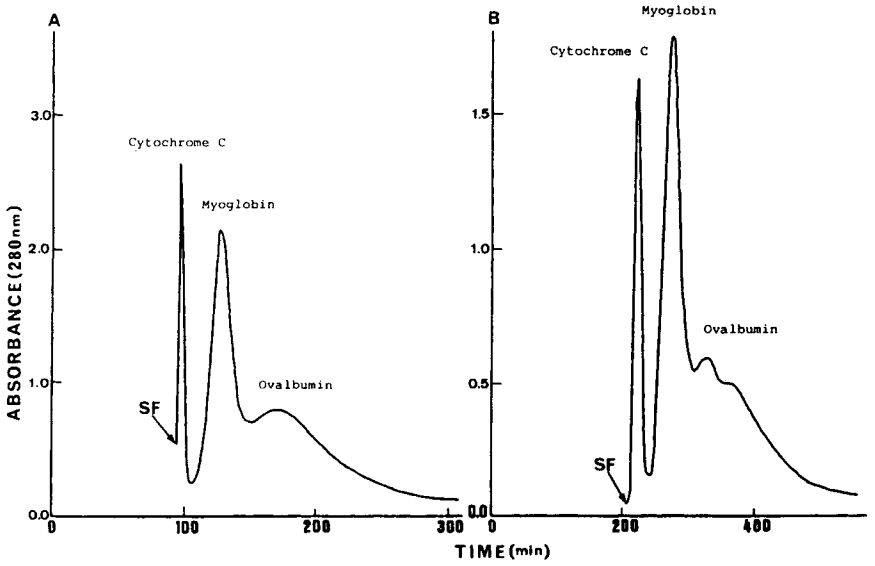

Figure 4. Chromatogram of a stable protein mixture obtained from analytical eccentric coil assemblies. Column: (A) 32 coil units with a total capacity of 35.4 mL, (B) 68 coil units with a total capacity of 64.5 mL; sample: (A) cytochrome C (3.6 mg), myoglobin (12 mg), and ovalbumin (Sigma) (45 mg), (B) cytochrome C (2.4 mg), myoglobin (8 mg), and ovalbumin (Sigma) (30 mg). Other conditions were same as those described in the Fig. 1 caption. SF = solvent front.

shoulder, showing the heterogeneity of the ovalbumin sample obtained from Sigma Chemical Co. This result suggests that the increased number of the coil unit and/or decrease in column ID will improve the partition efficiency of the eccentric coil assemblies for separating proteins.

CONCLUSIONS

The toroidal coil assemblies in the X-axis CPC produces a poor separation of the stable set of proteins with a polymer phase system. Better efficiencies can be achieved by the eccentric coil assemblies, especially in smaller diameter columns. The high efficiency separation of 450 TP has been attained using a 0.85 mm ID tubing wound onto 5 mm core with a total capacity of 64.5 mL.

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